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## Artikel Asli/Original Article

# Characteristics of Human Spermatozoa Harvested in Culture Media with and Without Serum Proteins (Ciri-ciri Spermatozoa Manusia yang Dituai dalam Media Kultur Bersama dan Tanpa Protein Serum)

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### ABSTRACT

*This study was aimed to determine the efficiency of synthetic protein-free media in spermatozoa washing, preparation and retention of the activity of washed spermatozoa over short periods in vitro. Normozoospermic semen samples (n = 71) were equally apportioned and washed using synthetic protein-free medium (PFM), minimum essential medium + HSA (MEM) or commercial protein-containing medium (CPC). Washed spermatozoa were cultured in vitro using PFM, MEM or CPC media and held for 24 hrs at 4°C, 15°C, 22°C or 37°C. Spermatozoa activity was evaluated at 0 hr, 4 to 7 hrs and 24 hrs post-wash. The effects of PFM on spermatozoa motility, vitality, membrane integrity and DNA fragmentation level were not significantly different from that of MEM and CPC media at 0 hr, 4 to 7 hrs and 24 hrs post-wash in vitro. Synthetic PFM, MEM and CPC retained spermatozoa activity highest when specimen were held at 22°C and it was significantly higher ( $p < 0.05$ ) than that at 37°C after 24 hrs incubation in vitro. However, no significant changes ( $p > 0.05$ ) were noted in spermatozoa DNA fragmentation (SDF) levels when specimen were held at 22°C or 37°C at 4 to 7 hrs and also after 24 hrs post-wash in vitro in all media. The use of synthetic PFM as an alternative to the commercial protein-containing media in human spermatozoa washing and preparation procedure for an efficient and safer (Assisted Reproduction Technology) ART outcome. Spermatozoa activity can be successfully retained at room temperature post-wash over short periods; spermatozoa may lose viability rapidly if held for long hours at 37°C in all media.*

**Keywords:** Protein-free medium; in-vitro culture; temperature; spermatozoa activity; DNA fragmentation

### ABSTRAK

*Kajian ini bertujuan untuk menentukan keberkesanan media bebas protein sintetik bagi pencucian spermatozoa, penyediaan dan penahanan aktiviti spermatozoa cucian dalam jangka masa terhad, secara in vitro. Sampel semen normozoospermik (n = 71) dibahagi-bahagikan secara sama rata dan dicuci dengan menggunakan medium bebas protein sintetik (PFM), medium esensial minimum + HSA (MEM) ataupun medium terkandung protein komersial (CPC). Spermatozoa cucian dikultur secara in vitro dengan menggunakan media PFM, MEM atau CPC dan ditahan selama 24 jam pada suhu 4°C, 15°C, 22°C atau 37°C. Aktiviti spermatozoa telah dinilai pada sela masa pascacucian 0 jam, 4 hingga 7 jam dan 24 jam. Kesan PFM terhadap motiliti, kevitatan, integriti membran dan tahap fragmentasi DNA tidak menunjukkan perbezaan signifikan berbanding kesan media MEM dan CPC pada sela masa 0 jam, 4 hingga 7 jam dan 24 jam pascacucian secara in vitro. PFM sintetik, MEM dan CPC mengekalkan aktiviti tertinggi bagi spermatozoa apabila spesimen ditahan pada suhu 22°C, dan aktiviti ini secara signifikan adalah lebih tinggi ( $p < 0.05$ ) pada suhu 37°C selepas inkubasi secara in vitro selama 24 jam. Walau bagaimanapun, tiada perubahan signifikan ( $p > 0.05$ ) diperhatikan terhadap tahap fragmentasi DNA spermatozoa (SDF) apabila spesimen ditahan pada suhu 22°C atau 37°C antara 4 hingga 7 jam dan juga selepas 24 jam proses pascacucian in vitro dilakukan dalam semua media. Penggunaan PFM sintetik sebagai alternatif kepada media terkandung protein komersial bagi pencucian spermatozoa manusia dan prosedur persediaan adalah untuk hasil ART (Assisted Reproduction Technology atau Teknologi Pembiakan Terbantu) yang lebih efisien dan selamat. Aktiviti spermatozoa boleh ditahan secara jayanya pada suhu bilik berikutan pascacucian dalam jangka masa yang pendek. Spermatozoa mungkin hilang kebolehhidupan dalam masa yang singkat sekiranya ditahan berjam-jam lamanya pada suhu 37°C dalam apa jua media.*

**Kata kunci:** Medium bebas protein; kultur in vitro; suhu; aktiviti spermatozoa; fragmentasi DNA

### INTRODUCTION

Assisted reproduction technology (ART) has witnessed an exponential growth and advances over the last four

decades. The number of children conceived through ART was reported to have reached 5 million in 2012 (Adamson et al. 2012). Over the years much concern has been expressed regarding the impact of ART treatment on health

of women and their babies one of which is the potential negative effects of undeclared constituents of the embryo culture and handling media.

One important consideration in spermatozoa preparation for ART procedures that is yet to be investigated in greater detail is the usefulness and efficacy of spermatozoa handling and culture media devoid of added donor human serum albumin (HSA). At the present moment most commercial culture media are supplemented with donor serum proteins (DSPs) which have been known as an essential component for spermatozoa survival (Critser et al. 1984) and needed for initiation of spermatozoa hyperactivation (Parrish et al. 1985). It has been reported that some patients were found to have gamete and embryo toxic factors in their serum (Dinkins et al. 2000 & Leveille et al. 1992). In addition, Alberda (1989) reported that pooled donor serum has a high risk of transmitting infections. There was also an incidence of potential transmission of vCJD in patients treated by and children born through ART (Kemmann et al. 1998). The risk of batch variation due to use of non-uniform serum protein supplements was raised as early as the 1980's (Leung et al. 1984). Beside the proteins a variety of other contaminants including numerous undeclared or unknown components are also present in the DSPs, which varies with each batch creating batch to batch variation and inconsistency in the content of the culture media (Alvarez et al. 2003). The European Union recommends the avoidance of non-uniform biological components (e.g., HSA) in healthcare products (EU Tissue Directive No. 2004/23/EU). The protein-free media (PFM) is a synthetic media devoid of HSA which, in contrast to other media, is considered safer, as the potential for diseases transmission is negligible.

Spermatozoa quality and the level of DNA fragmentation at the time of insemination have a significant impact on subsequent normal embryo and foetal development (Alvarez et al. 2003 & Morris et al. 2002). It is vital to keep washed spermatozoa viable, motile and intact long after preparation until the time of insemination (Agarwal et al. 2004). The present investigation aims to demonstrate the efficacy of the PFM for washing and preparation of human spermatozoa as well as for maintaining the quality of washed spermatozoa *in vitro* over extended periods of time, which is a useful attribute in ART since insemination normally occurs a couple of hours after spermatozoa preparation.

## MATERIALS AND METHODS

### SEMEN COLLECTION AND ANALYSIS

Normozoospermic semen samples were obtained from 71 men undergoing semen assessment at the University Malaya Fertility Centre (UMFC), the Polyclinic at the University Malaya Medical Centre (UMMC) and the infertility unit of Kuala Lumpur Hospital (HKL) from August 2014 through January 2015. Ethics/IRB approval was obtained (Ref. 1073.52). A written informed consent

was collected from each patient who agreed to participate in the study. Semen samples were obtained from the participants after they have abstained for 2-3 days from sexual activity. Semen was produced by masturbation in a private room close to the laboratory. Semen analysis was performed according to the WHO 2010 standard format (Beatty 1964; WHO 2010).

### SPERMATOZOA WASHING AND PREPARATION

Semen samples were washed by the standard density gradient centrifugation (DGC) technique (Bhattacharya 1958; Bolton et al. 1984; Morrell et al. 2009; Schilling 1966) as per the instructions given kits provided by the manufacturers (GradiART Upper and lower layers, Cat. 200150 and 300150, *Cellcura*<sup>TM</sup> ASA, Norway).

**Experiment 1** Semen samples were washed using different washing media: Synthetic Protein-Free Medium (PFM, Cat. EM100150, *Cellcura* ASA, Norway), Minimum Essential Medium + HSA (MEM, Biowest SAS, France), or Commercial Protein-Containing Media (CPC, Irvine Scientific, USA). Sibling spermatozoa were subsequently cultured *in vitro* using PFM, MEM or CPC culture media for 24 hrs. Spermatozoa activity was evaluated at 0 hr, 4 to 7 hrs and after 24 hrs post-wash.

**Experiment 2** (a) The motility of washed spermatozoa held at 4°C, room temperature (22°C) and 37°C was evaluated in PFM and compared with that of sibling spermatozoa cultured in MEM and CPC culture media after 4 to 7 hrs and 24 hrs post-wash. (b) An additional holding temperature of 15°C was included while all other conditions remained constant as in Experiment 2(a).

**Experiment 3** Spermatozoa vitality, membrane integrity and the DNA fragmentation (SDF) levels at 22°C (optimal temperature as obtained in Experiment 2) were compared to that held at 37°C (Control) using PFM which was also evaluated and compared to sibling spermatozoa cultured using MEM and CPC culture media after 4 to 7 hrs and 24 hrs post-wash. Prior to incubation and after each examination, samples of washed spermatozoa were equilibrated for 5 mins inside the incubator with a gas mixture (6% CO<sub>2</sub>) in order to maintain the pH of the culture medium.

### EVALUATION OF SPERMATOZOA MOTILITY AND VITALITY

A modified *Neubauer Chamber* (Hawksley, Lancing, England) was used to assess spermatozoa motility. Observations were made using a light microscope at 200 × magnification. Readings were expressed as percentage of total motility. Due to the propensity of spermatozoa heads to stick to the surface of the counting chamber, a holding time of 5 to 7 mins was required after charging the Neubauer chamber to enable the spermatozoa to detach

themselves as recommended by the Inventor of the PFM (one of us: JA). It is noted that this behavior was observed only in PFM but not in CPC and MEM as was also reported by Peirce (Peirce et al. 2015). Therefore, all samples were held for 5 to 7 mins prior to the motility evaluation in order to standardize the method and render our treatment identical for all specimens irrespective of the washing or culture media used. *Spermatozoa VitalStain*<sup>TM</sup> (Nidacon International AB, Mölndal, Sweden) was used to give an indication of live and dead spermatozoa in each treatment. The VitalStain<sup>TM</sup> solution contains both eosin and nigrosine stains. Equal amounts of the VitalStain<sup>TM</sup> solution and spermatozoa sample (50 µl svb + 50 µl spermatozoa sample) were mixed well in an Eppendorf micro tube. The mixture was kept for 30 sec at room temperature prior to observation. A total of 200 spermatozoa were counted per observation.

#### EVALUATION OF SPERMATOZOA PLASMA MEMBRANE INTEGRITY

The hypo-osmotic swelling (HOS) test was performed as described by Jeyendran (Jeyendran et al. 1984). It is suggestive of the functionality and integrity of spermatozoa plasma membrane and thus an assumption of its viability. Spermatozoa were treated with a hypo-osmotic solution. A modified Neubauer Chamber (Hawksley, Lancing, England) was filled with the treated spermatozoa and allowed to stand for at least 1 min before observations were made under the phase-contrast microscope at 400 × magnification. A total of 200 spermatozoa were observed for swellings. Membrane Integrity Level was calculated using Eqn. (1).

$$\text{Membrane Integrity Level (\%)} = \frac{\text{Number of Spermatozoa with Swollen Tails} \times 100}{\text{Total Number of Spermatozoa Counted}} \quad (1)$$

The response to the hypo-osmotic solution was considered when swollen or a small enlargement present at the tip of the tail or at the junction of the midpiece. The response was also considered in cases when the tail is curved, shortened or thickened as previously described (Jeyendran et al. 1984).

#### EVALUATION OF SPERMATOZOA DNA FRAGMENTATION

Spermatozoa DNA fragmentation (SDF) level was measured using the Halosperm® G2 kit (Halotech DNA, Madrid, Spain). The assay was performed according to the instructions of the manufacturer. The presence of a halo of chromatin decondensation around the head of the

treated spermatozoa is an indication of their DNA integrity. In contrast, spermatozoa with no halo are considered fragmented DNA. Observations were made under the bright field light microscope at 400 × magnification. A total of 200 spermatozoa were counted for each treatment. Readings were expressed as percentage of spermatozoa with intact DNA. A negative control was performed alongside with each sample tested. Sibling spermatozoa of the control were not treated with denaturing solution, therefore all spermatozoa of intact and fragmented DNA responded to the stains and showed a halo of chromatin dispersion around the head. Spermatozoa DNA Integrity Level was calculated using Eqn. (2).

$$\text{Spermatozoa DNA Integrity Level (\%)} = \frac{\text{Number of Spermatozoa with Halo} \times 100}{\text{Total Number of Spermatozoa Counted}} \quad (2)$$

#### STATISTICAL ANALYSIS

Statistical analysis was performed using the IBM SPSS Statistical Software Version 22 (International Business Machines Corp, New York 10504). The results are presented as means of proportions. The data obtained were homogenous and normally distributed. The effects of culture media and holding temperature on various spermatozoa activities were compared through ANOVA. The differences between groups of variables were calculated through the post-hoc test (two-way ANOVA/Duncan). Pairwise, comparisons between two means of various parameters, were determined using the *paired t-test*.

#### RESULTS

##### EXPERIMENT 1: ACTIVITY EVALUATION OF HUMAN SPERMATOZOA WASHED AND CULTURED USING PFM, MEM OR CPC MEDIA

There were no significant differences between PFM, MEM and CPC handling media with regard to spermatozoa motility ( $p = 0.206$ , ns); vitality ( $p = 0.315$ , ns); plasma membrane integrity ( $p = 0.401$ , ns) and DNA integrity level ( $p = 0.587$ , ns) at 0 hr post-wash (Figure 1). After 4 to 7 hrs of *in vitro* culture no significant differences were noted in the levels of spermatozoa motility ( $p = 0.891$ , ns), vitality ( $p = 0.848$ , ns), plasma membrane integrity ( $p = 0.134$ , ns) and DNA integrity level ( $p = 0.182$ , ns) obtained using PFM,

MEM or CPC culture media (Figure 2 (a)). Likewise, after 24 hrs, the effect between the media was not significant when the same parameters were evaluated; motility ( $p$

$= 0.490$ , ns), vitality ( $p = 0.662$ , ns), plasma membrane integrity ( $p = 0.557$ , ns) and DNA integrity level ( $p = 0.661$ , ns) (Figure 2 (b)).

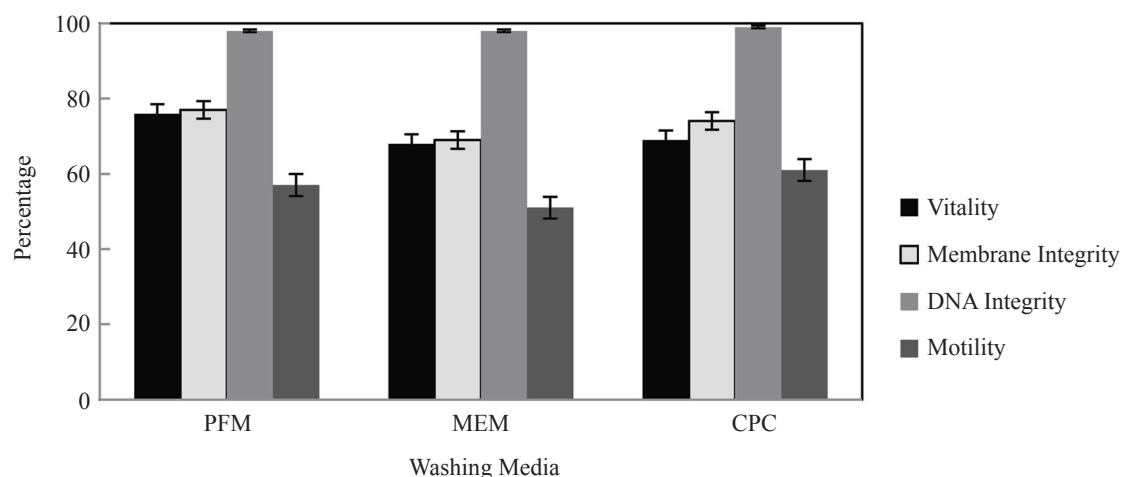


FIGURE 1. Effect of protein-free medium (PFM), minimum essential medium (MEM) and commercial protein-containing washing medium (CPC) on spermatozoa vitality, plasma membrane integrity, DNA integrity level and motility of human spermatozoa at 0hr post-wash. Results obtained from 25 men ( $n = 25$ ). No significant difference ( $p > 0.1$ ) between the parameters of spermatozoa washed using different washing media

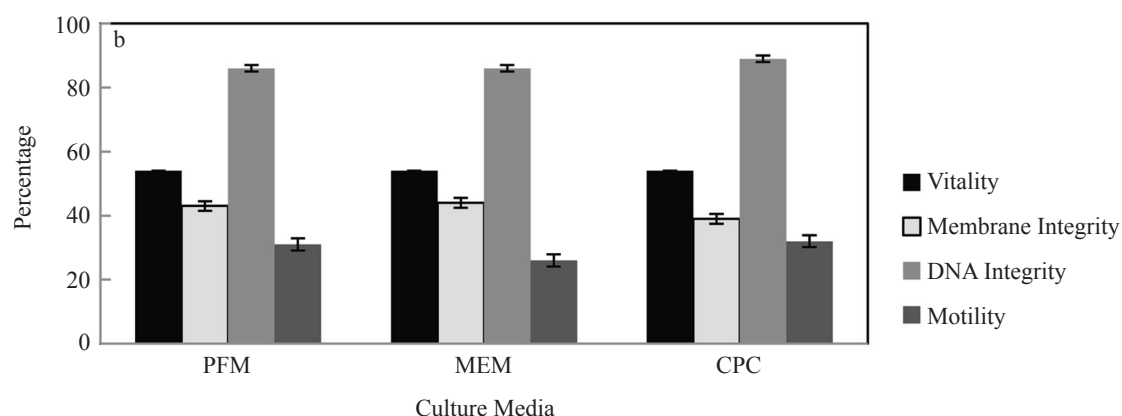
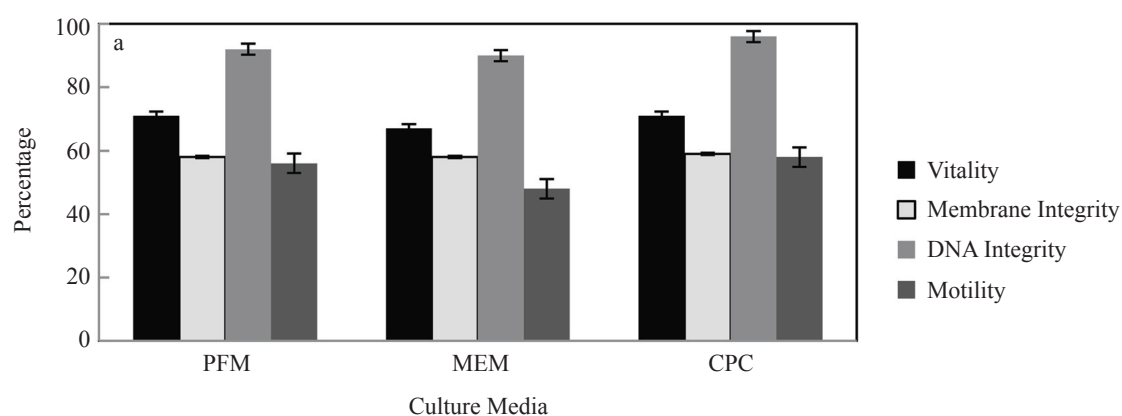


FIGURE 2. Effect of protein-free medium (PFM), minimum essential medium (MEM) and commercial protein-containing culture medium (CPC) on the vitality, plasma membrane integrity, DNA integrity level and motility of washed human spermatozoa after 4 to 7 hrs (a) and 24 hrs (b) post-wash *in vitro* under a gaseous phase of 6% CO<sub>2</sub> in the incubator at 37°C. Results obtained from 25 men ( $n = 25$ ). No significant difference ( $p > 0.1$ ) between the parameters of spermatozoa cultured in different media.



EXPERIMENT 2: MOTILITY EVALUATION OF HUMAN SPERMATOZOA HELD FOR 24 HRS POST-WASH AT 4°C, 15°C, 22°C AND 37°C USING PFM, MEM AND CPC CULTURE MEDIA

The findings of Experiment 2(a) and (b) revealed that after 4 to 7 hrs the highest spermatozoa motility was obtained at 22°C, which was significantly higher than that at 4°C ( $p < 0.05$ ), but  $p > 0.05$  when compared to that at 15°C and 37°C in PFM, MEM and CPC culture media. However, 24 hrs post-wash, spermatozoa motility at 22°C remained the highest ( $p < 0.05$ ) compared to those at 4°C, 15°C and 37°C in all culture media (Tables 1 and 2). At 4 to 7 hrs of *in vitro* holding duration, spermatozoa motility at 22°C appeared to be not significantly different from the initial motility recorded at 0 hr post-wash. However, a significant difference was shown when the motility of spermatozoa cultured at 4°C, 15°C and 37°C was compared individually with the initial motility. On the other hand, after 24 hrs, spermatozoa motility at all pre-set temperatures was significantly reduced when compared to that at 0 hr post-wash in all culture media.

In Table 1, results are expressed as mean of motility percentage, (%) = (No. of motile spermatozoa/( $n = 200$ )  $\times 100$ ). The mean values of different superscripts (a,d,c) in each column at each point of holding time are significantly different. (\*) significantly different, when the motility at 4 to 7 hrs & 24 hrs were compared individually with the initial motility (0 hr) in all culture media. The mean values in each row are not significantly different when compared to the other rows of different culture media.

TABLE 1. Effect of culture media and holding temperature (4°C, 22°C and 37°C) on washed spermatozoa motility *in vitro*

Culture Media	Holding Time/ Hour	37°C	22°C	4°C
Protein-Free Medium (PFM) ( $n = 25$ )	0 hr	54 <sup>(a)</sup>	54 <sup>(a)</sup>	54 <sup>(a)</sup>
	4-7 hrs	41 <sup>(a,b)*</sup>	47 <sup>(b)</sup>	39 <sup>(a)‡</sup>
	24 hrs	23 <sup>(c)‡</sup>	45 <sup>(b)†</sup>	12 <sup>(a)‡</sup>
Minimum Essential Medium (MEM) ( $n = 25$ )	0 hr	50 <sup>(a)</sup>	50 <sup>(a)</sup>	50 <sup>(a)</sup>
	4-7 hrs	43 <sup>(a,b)</sup>	45 <sup>(b)</sup>	34 <sup>(a)</sup>
	24 hrs	8 <sup>(c)</sup>	35 <sup>(b)</sup>	5 <sup>(a)</sup>
Commercial Protein- Containing Medium (CPC) ( $n = 25$ )	0 hr	59 <sup>(a)</sup>	59 <sup>(a)</sup>	59 <sup>(a)</sup>
	4-7 hrs	49 <sup>(a,b)</sup>	55 <sup>(b)</sup>	41 <sup>(a)</sup>
	24 hrs	27 <sup>(c)</sup>	51 <sup>(b)</sup>	14 <sup>(a)</sup>

TABLE 2. Effect of culture media and holding temperature (4°C, 15°C, 22°C and 37°C) on washed spermatozoa motility *in vitro*

Culture Media	Holding Time/ Hour	37°C	22°C	15°C	4°C
Protein-Free Medium (PFM) ( $n = 25$ )	0 hr	52 <sup>(a)</sup>	52 <sup>(a)</sup>	52 <sup>(a)</sup>	52 <sup>(a)</sup>
	4-7 hrs	40 <sup>(a,b)*</sup>	47 <sup>(b)</sup>	41 <sup>(a,b)*</sup>	36 <sup>(a)</sup>
	24 hrs	22 <sup>(b)</sup>	43 <sup>(c)*</sup>	33 <sup>(b)</sup>	15 <sup>(a)</sup>
Minimum Essential Medium (MEM) ( $n = 25$ )	0 hr	48 <sup>(a)</sup>	48 <sup>(a)</sup>	48 <sup>(a)</sup>	48 <sup>(a)</sup>
	4-7 hrs	41 <sup>(a,b)</sup>	44 <sup>(b)</sup>	38 <sup>(a,b)</sup>	33 <sup>(a)</sup>
	24 hrs	18 <sup>(b)</sup>	37 <sup>(c)</sup>	28 <sup>(b)</sup>	8 <sup>(a)</sup>
Commercial Protein- Containing Medium (CPC) ( $n = 25$ )	0 hr	57 <sup>(a)</sup>	57 <sup>(a)</sup>	57 <sup>(a)</sup>	57 <sup>(a)</sup>
	4-7 hrs	49 <sup>(a,b)</sup>	55 <sup>(b)</sup>	49 <sup>(a,b)</sup>	40 <sup>(a)</sup>
	24 hrs	27 <sup>(b)</sup>	49 <sup>(c)</sup>	41 <sup>(b)</sup>	12 <sup>(a)</sup>

In Table 2, results are expressed as mean of motility percentage, (%) = (No. of motile spermatozoa/( $n = 200$ )  $\times 100$ ). The mean values of different superscripts (a,d,c) in each column at each point of holding time are significantly different. (\*) significantly different when the motility at 4 to 7 hrs & 24 hrs were compared individually with the initial motility (0 hr) in all culture media. The mean values in each row are not significantly different when compared to the other rows of different culture media.

EXPERIMENT 3: EVALUATION OF VITALITY, PLASMA MEMBRANE INTEGRITY AND DNA INTEGRITY OF SPERMATOZOA HELD AT 22°C FOR 24 HRS POST-WASH USING PFM, MEM AND CPC CULTURE MEDIA

Spermatozoa vitality at 22°C in PFM, MEM and CPC was significantly higher than that at 37°C after 4 to 7 hrs and 24 hrs post-wash. However, when the vitality at both 22°C and 37°C were compared individually with the initial vitality level obtained at 0 hr post-wash, a significant reduction was noted in all media at 4 to 7 hrs and also after 24 hrs of *in vitro* holding duration (Figure 3 (a)). The integrity levels of spermatozoa plasma membrane were likewise significantly higher at 22°C than those at 37°C in PFM, MEM and CPC after 4 to 7 hrs and 24 hrs post-wash. At 4 to 7 hrs, the membrane integrity at 22°C was found to be not significantly different from the initial levels obtained at 0 hr post-wash in PFM and CPC culture media, but it was significantly reduced in MEM culture media. In contrast, after 24 hrs a significant reduction in the level of membrane integrity of spermatozoa held at 22°C was noted in comparison to the initial readings in PFM, MEM and CPC culture media (Figure 3 (b)). There was no significant difference in the SDF level when spermatozoa were held at room temperature (22°C) or 37°C up to 24 hrs post-wash in all culture media. Spermatozoa DNA integrity level at 22°C was not significantly different when compared to the initial DNA integrity level obtained at 0 hr post-wash, in PFM, MEM and CPC (Figure 3 (c)).

## DISCUSSION

The present investigation focused on determining the efficiency of protein-free washing and culture media in the preparation of human spermatozoa for application in assisted reproduction techniques.

The findings of this study suggest that in general, the protein-free media are equally efficient as the commercial protein-containing media and it is capable of retaining spermatozoa activity and viability over short periods post-wash. The synthetic media devoid of added donor HSA are considered safer and disease-free as the potential for disease transmission is negligible compared to those with donor serum proteins. Other studies have shown the fertilizing ability of spermatozoa prepared in PFM is not impaired when applied to conventional IVF and ICSI techniques (Ali et al. 2000 & Ali 2004). Furthermore, a

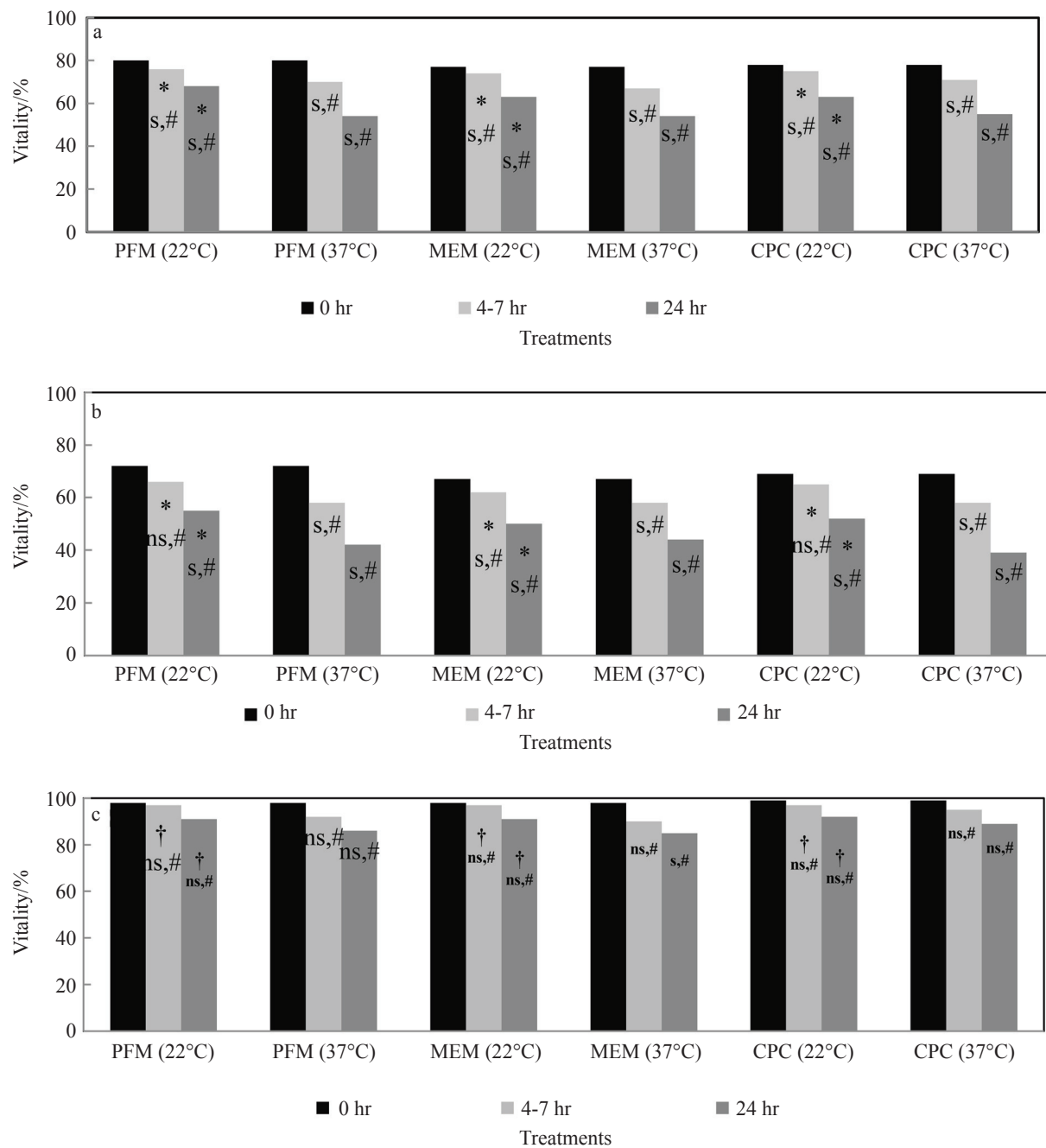


FIGURE 3. Effect of protein-free medium (PFM), minimum essential medium (MEM), commercial protein-containing (CPC) culture medium and holding temperature (22°C & 37°C) on washed spermatozoa vitality (3(a)), membrane integrity level (3(b)) and DNA integrity level (3(c)) at 4 to 7 hrs and 24 hrs *in vitro* incubation. Results obtained from 21 men ( $n = 21$ ). (\*)  $p < 0.05$ ; (†)  $p > 0.05$  when the level at 22°C was compared individually with the control (37°C) in all culture media. (s)  $p < 0.05$ ; (ns)  $p > 0.05$  when the levels at 4 to 7 hrs and 24 hrs were compared individually with the initial levels obtained at 0 hr post-wash in all culture media. (#)  $p > 0.05$  when spermatozoa vitality, membrane integrity and DNA integrity levels were compared at different culture media.

multicenter IUI clinical trial performed with spermatozoa prepared with Cellcure™ synthetic protein-free medium (PFM) resulted in a clinical pregnancy rate of 26% as was reported by Cellcure ASA, Norway, online [www.cellcure.com] (Cellcure ASA online 2015). In addition, as a chemically defined synthetic medium, the PFM has the added advantage of being free of batch to batch variations, unlike media containing donor serum proteins that are

predisposed to batch variations. The synthetic media therefore, allow for safer ART outcome and better quality control in ART laboratories.

The present study demonstrated a significantly better and longer retention of spermatozoa motility when washed samples were incubated at room temperature (22°C) for 24 hrs compared to the control held at 37°C, and at other holding temperatures in both protein-free

and protein-containing culture media. This finding was supported by the observations of Thijssen (Thijssen et al. 2014) who observed better spermatozoa quality in washed spermatozoa samples incubated at room temperature than in those incubated at 37°C. The attempt to retain optimal spermatozoa motility over a short period of time by reducing the holding temperature to 15°C proved to be of no benefit. The results of this study are in agreement with other investigations (Schuffner et al. 2002; Dougherty et al. 1975) in which a significant loss of spermatozoa motility and an increased incidence of apoptosis were observed when washed specimen was incubated at 37°C over extended periods of time. In another study (Calamera et al. 2001) major alterations in spermatozoa functions and a significant reduction in the number of motile spermatozoa together with impairment to spermatozoa motility were noted during prolonged *in vitro* incubation at 37°C with 5% CO<sub>2</sub> in. The present findings are also similar to the observations of other workers (Annelies et al. 2014) where a significantly higher retention of spermatozoa motility and viability were obtained when samples were incubated at room temperature compared to 35°C (testis temperature).

Furthermore, the present findings are comparable to the observations of Esfandiari and co-workers who reported a significantly lower motility in washed human spermatozoa at 4°C compared to 37°C and 25°C (Esfandiari et al. 2002). The unique findings of this study are the novel approach of preparing spermatozoa in a synthetic PFM medium without loss of spermatozoa viability and activity. Another important advantage of the PFM is the observation that the synthetic PFM behaves in a manner identical to or better than conventional protein-containing media when it comes to retention of spermatozoa activity after preparation, especially over a period of time. In conclusion these findings support the authors' assumption that the synthetic PFM to be a useful, safe and chemically defined alternative to the conventional protein-containing media which is prone to batch variation, is potentially hazardous, and is chemically undefined.

At 4 to 7 hrs post-preparation, no significant changes were observed in spermatozoa motility when held at 15°C, 22°C and 37°C. However, after 24 hrs, spermatozoa held at 22°C showed a significantly highest motility compared to the other treatments. These findings are comparable to previous studies (Jackson et al. 2010; Lachaud et al. 2004; Petrella et al. 2014) where a significant change in the total motility at room temperature was only noted after 24 hrs of *in vitro* holding duration. In addition, another study (Marin-Briggiler et al. 2002) indicated that when human spermatozoa are incubated at room temperature, they assume a resting state, which allows them to preserve their energy and quality.

Additionally, the findings of Marin-Briggiler (Marin-Briggiler et al. 2002) revealed that the incubation of human spermatozoa at around room temperature led to protein

tyrosine phosphorylation patterns similar to that of non-capacitated spermatozoa. This finding is significant in the present context, because it suggests that by holding washed spermatozoa at room temperature, undesired premature capacitation that leads to loss of viability with time can be avoided until the time of insemination. These observations are also in agreement with the findings in hamsters (Si 1999). On the other hand, Gallup proposed 'the activation hypothesis' as a mechanism of spermatozoa capacitation *in vivo* (Gallup 2009). In contrast to this condition prevalent in the female reproductive tract, human spermatozoa held at room temperature *in vitro* can be activated by raising the temperature to 37°C (Eisenbach et al. 1999). However, both hyperactivation and capacitation do not last long, only between 50 mins to 4 hrs (Chavarria 1992) then spermatozoa will lose its motility and start to die. These findings explain the diminished survival of spermatozoa held at 37°C compared to those held at lower temperatures and possibly the persistently low pregnancy rate in IUI treatment procedures, when insemination was performed many hours before ovulation.

Human spermatozoa vitality was retained highest at 22°C when compared with the control (body temperature) in both protein-free and protein-containing culture media at 4 to 7 hrs and also after 24 hrs post-wash. This finding is in agreement with that of Aitken (Aitken et al. 1996) who showed that spermatozoa incubation at ambient temperatures (i.e. 22°C) had no effect on their vitality or their potential to undergo acrosome reaction. In contrast, the present work revealed that the proportion of spermatozoa held at 22°C has higher plasma membrane integrity level than that at 37°C. Therefore, this suggests that spermatozoa vitality and plasma membrane integrity may perhaps involve the same enzymatic activities, which are optimal at room temperature (22°C). It has also been suggested that these parameters are related to the capacitation and acrosome reactions, where capacitated spermatozoa tend to become non-viable and die faster (Dalzell et al. 2004). The practice of short term conservation of spermatozoa at room temperature post-preparation could prevent or block the early capacitation process and preserve the integrity and viability of washed spermatozoa long after its preparation.

Furthermore, this study showed that holding spermatozoa at room temperature does not have any adverse effect on the DNA integrity level up to 24 hrs post-preparation in all media. The DNA integrity level after 24 hrs post-wash at RT was not significantly different with the original DNA integrity level obtained at 0 hr post-preparation. On the contrary, it was demonstrated that the prolonged incubation of normozoospermic samples at 37°C is associated with higher rates of spermatozoa DNA fragmentation (Agarwal et al. 2016; Nabi et al. 2014). Furthermore, Matsuura (Matsuura et al. 2010) who showed that spermatozoa DNA fragmentation was significantly lower in samples incubated at room temperature compared

to those incubated at 37°C. This suggests that body temperature has a negative effect on the spermatozoa DNA integrity, which is further strengthened by the observations of Hammadeh (Hammadeh et al. 2001) which recorded a significant increase in uncondensed chromatin from 25-91% when spermatozoa was incubated for 24 hrs at 37°C.

The present study indicated that, DNA fragmentation index (DFI; % of DNA damaged spermatozoa) of spermatozoa held for 4 to 7 hrs at RT is 3% and increased to about 9% after 24 hrs incubation in all culture media. It has been suggested that if the proportion of DFI is less than 15%, the chance of fertilization is excellent and the DFI of 15-30% predicts a good fertility potential. However, above 30% is an indicative of fair to poor fertility potential (Guerin et al. 2005). Based on this suggestion we assume that holding spermatozoa at RT may have an excellent fertility potential even if held for up to 24 hrs *in vitro* in a pH controlled culture media. Previously, we have noted in our fertility clinic that the use of spermatozoa prepared in PFM held at 22°C resulted in a fertilization rate of 75.8% (n = 30), positive  $\beta$ -hCG pregnancy rate of 53.3% (n = 30), clinical pregnancy rate is 43.3% (n = 30) and the live birth rate is 33% (n = 30) following routine intracytoplasmic spermatozoa injections (ICSI). This observation serves as the proof of principle for the technique of holding washed human spermatozoa in protein-free media at room temperature over short periods of time prior to ART insemination.

The minimum essential media (MEM) supplemented with HSA have shown no significant difference when compared to the specialized ART media in the effectiveness of spermatozoa washing, culture and short term conservation *in vitro* at 22°C. However, it was shown in this study that the MEM media was not able to support the membrane integrity and the DNA integrity level after 24 hrs post-wash culture of washed spermatozoa held at 37°C *in vitro*, when compared to the sibling spermatozoa cultured in PFM and CPC culture media. This suggests that MEM washing and culture media can be used for washing and short term conservation only *in vitro* as a replacement for the specialized media, especially in cases where the ART centers are far away from the manufacturer or in cases where the specialized media have failed quality control.

The ability to use protein-free media to hold prepared spermatozoa up to 24 hrs at ambient temperature (22°C) without loss of viability and DNA integrity would be useful for making ART procedures safe and more efficient, which can be also beneficial for the transportation of washed specimens to other local centers for diagnostic or ART treatment services.

## CONCLUSION

The synthetic protein-free washing and culture media are equally efficient as the conventional protein-containing

media. Human spermatozoa held at room temperature can be successfully retained with significant retention of their motility and viability up to 24 hrs post-preparation using protein-free media. The present findings suggest that laboratories change their routine of holding washed spermatozoa from 37°C to room temperature (22°C) and to use safe media devoid of added donor serum for spermatozoa preparation, in order to make ART safe, maximize the potential of the inseminating spermatozoa and to prevent impairment.

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